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Current Topics

Novel Antisense and Peptide Nucleic Acid Strategies for Controlling Gene Expression†

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ABSTRACT: Antisense oligonucleotides have the potential to make revolutionary contributions to basic science and medicine. Oligonucleotides can bind mRNA and inhibit translation. Because they can be rapidly synthesized to be complementary to any sequence, they offer ideal tools for exploiting the massive amount of genome information now available. However, until recently, this potential was largely theoretical, and antisense experiments often produced inconclusive or misleading outcomes. This review will discuss the chemical and biological properties of some of the different types of oligomers now available and describe the challenges confronting in vitro and in vivo use of oligonucleotides. Oligomers with improved chemical properties, combined with advances in cell biology and success in clinical trials, are affording powerful new options for basic research, biotechnology, and medicine.

In theory, using oligonucleotides to inhibit gene expression should be simple. A target sequence is chosen and its complement synthesized. The oligomer is introduced into cells, binds mRNA, and reduces translation. Oligonucleotide-mediated inhibition would nicely complement other approaches for studying cellular processes and offer an important tool for investigating the details of signaling, disease progression, and differentiation. In practice over the past decade, however, gene inhibition by antisense oligomers has not proven to be a robust or generally reliable technology. Many researchers are skeptical about the approach, and it has been suggested that many published studies are at least partially unreliable (*1*).

Why has such a simple and potentially powerful strategy not been a routine tool for investigating the cellular function of proteins? One answer is that it has been difficult to identify oligonucleotides that act as potent inhibitors of gene expression, primarily due to difficulties in predicting the secondary structures of RNA. Due to the secondary structures of RNA there are a limited number of freely accessible regions which often creates a situation whereby it may be necessary to screen 20 or more oligomers before identifying one that functions adequately (*2*). Another answer is that even when active oligomers are discovered, the difference in oligonucleotide dose required to inhibit expression is often not much different than doses that lead to nonselective toxicity and cell death. Finally, oligonucleotides can bind to proteins and produce artifactual phenotypes that obscure effects due to the intended antisense mechanism (*3*).

The need for antisense oligomers that are more potent and more selective has been widely recognized and has led to the development of chemical modifications to improve binding and selectivity. Gene inhibition has also been improved by exploiting the ability of oligonucleotides to recruit cellular enzymes that assist the recognition and destruction of mRNA (*4*−*6*). In this review, we will first

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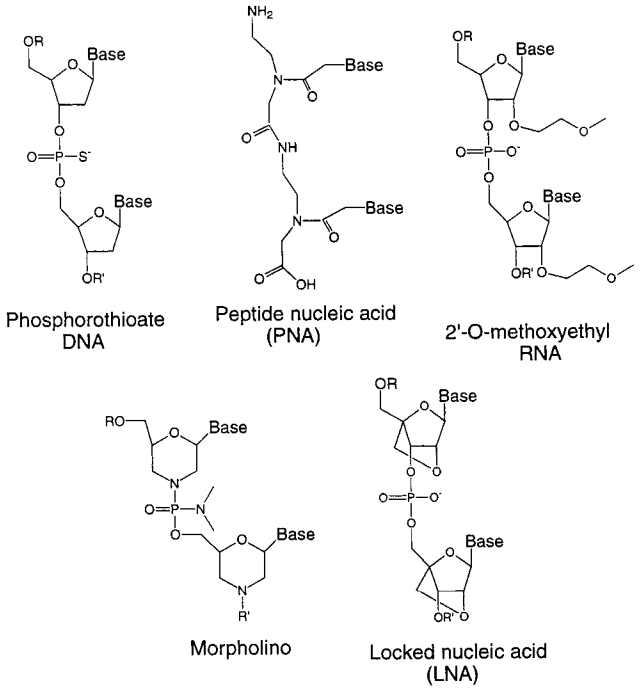

FIGURE 1: Nucleic acid analogues and chemical modifications mentioned in this review: phosphorothioate DNA, 2′-*O*-methoxyethyl RNA, morpholino, peptide nucleic acid (PNA), and locked nucleic acid (LNA).

describe chemical properties of oligonucleotides and oligonucleotide mimics that contribute to the potency and specificity of antisense oligomers. We will then summarize the substantial recent progress in the application of antisense and antigene oligomers for functional genomics and drug development. Finally, we will conclude by describing several novel strategies for using oligonucleotides to control gene expression.

IMPORTANT PROPERTIES OF ANTISENSE OLIGOMERS

Phosphorothioate Linkages. The introduction of phosphorothioate (PS)[1] linkages (Figure 1) profoundly influences the properties of antisense oligonucleotides. Originally, PS linkages were intended to enhance the nuclease resistance of oligonucleotides (*7*), but they do much more than simply slow degradation. PS linkages also improve pharmacokinetic properties by promoting binding to serum proteins, greatly increasing in vivo half-life, thereby facilitating the development of oligonucleotide drugs (*8*).

Enhanced protein binding upon introduction of PS linkages can present problems for the interpretation of antisense experiments. Interactions between PS oligonucleotides and proteins (*3*) can lead to misleading phenotypes that obscure effects due to sequence-specific recognition of a nucleic acid target. Such unintended interactions account for many of the difficulties that have slowed antisense research in the past. Fortunately, the likelihood of misleading can be minimized by use of mismatch and scrambled control oligonucleotides (*9*) and by avoiding G-rich oligomers that can form quadruplex secondary structures (*10*). These are not the only precautions that can be taken, and Crooke (10) provides detailed criteria for sound experimental design.

Increased Affinity of Hybridization. One strategy for improving the efficacy of antisense oligonucleotides is to increase the difference between their affinity for their intended targets and their propensity to bind to nontargeted molecules. Many modifications are available to enhance the affinity of oligonucleotide binding, but modification of the 2′-hydroxyl of RNA (Figure 1) is a relatively conservative change that has been the focus of much research. 2′-Modified RNA cannot act as a substrate for RNase H, and if cleavage of mRNA is desired, it is necessary to include a central DNA window containing at least six DNA bases (*11*, *12*). 2′-*O*-Methyl- and 2′-methoxyethyl-modified RNAs are viewed as "second generation" antisense molecules (the first generation being uniformly labeled PS-DNA) and are currently being tested in clinical trials.

Uptake by Cultured Cells and Tissue Distribution in Animals. Oligonucleotides do not efficiently enter most types of cultured cells when added alone, and to achieve a useful level of uptake, it is usually necessary to employ a commercially available cationic lipid or other transfection reagent (*13*). Transfection conditions will vary depending on cell line and will need to be optimized for each new experiment. Many transfection reagents are available, and empirical testing is required to identify the best choice for transfecting a given cell line.

In contrast to the situation in cell culture, uncomplexed oligonucleotides that contain phosphorothioate linkages spontaneously enter some tissues when introduced intravenously (*8*). Delivery to the liver and kidney is most efficient, but the spleen, intestine, and other organs also receive significant doses (*8*). Promising data from ongoing clinical studies also suggest that oligomers can enter human tumors upon intravenous administration and produce a therapeutic effect (*8*, *14*−*16*). Oligonucleotides exhibit some oral bioavailability, and this may prove a useful route for clinical administration in the future (*17*). An important consideration for in vivo use is that oligonucleotides containing CpG motifs can stimulate the immune system (*18*). While this stimulation may be advantageous for some therapies, it might also lead to non-antisense effects and misleading results. The immunostimulatory effect of cytidine can be negated by use of 4-methylcytosine and other cytosine derivatives.

Recruitment of Cellular Enzymes. Depending on where they are targeted within mRNA, antisense oligonucleotides can use several mechanisms to block or alter gene expression (Figures 2 and 3). When targeted to the terminus of the 5′ untranslated region, oligomers can prevent ribosome binding, while oligomers targeted throughout the transcript may be able to act as a roadblock to peptide elongation (Figure 2A,B). Oligomers may also be able to block splice sites and alter the production of splice variants (Figure 2C). In these three mechanisms, the mRNA remains intact, and the efficacy of the approach can be followed by observing diminished or altered expression of protein.

Oligonucleotides can recruit cellular enzymes to degrade target mRNAs (Figure 3). mRNA cleavage can amplify the efficacy of antisense oligomers, and the ability or inability of oligonucleotides to recruit cellular enzymes and promote cleavage of mRNA differentiates competing antisense approaches. Oligomers that contain a contiguous stretch of at

[1] Abbreviations: PS, phosphorothioate; PNA, peptide nucleic acid; LNA, locked nucleic acid; RNAi, RNA interference.

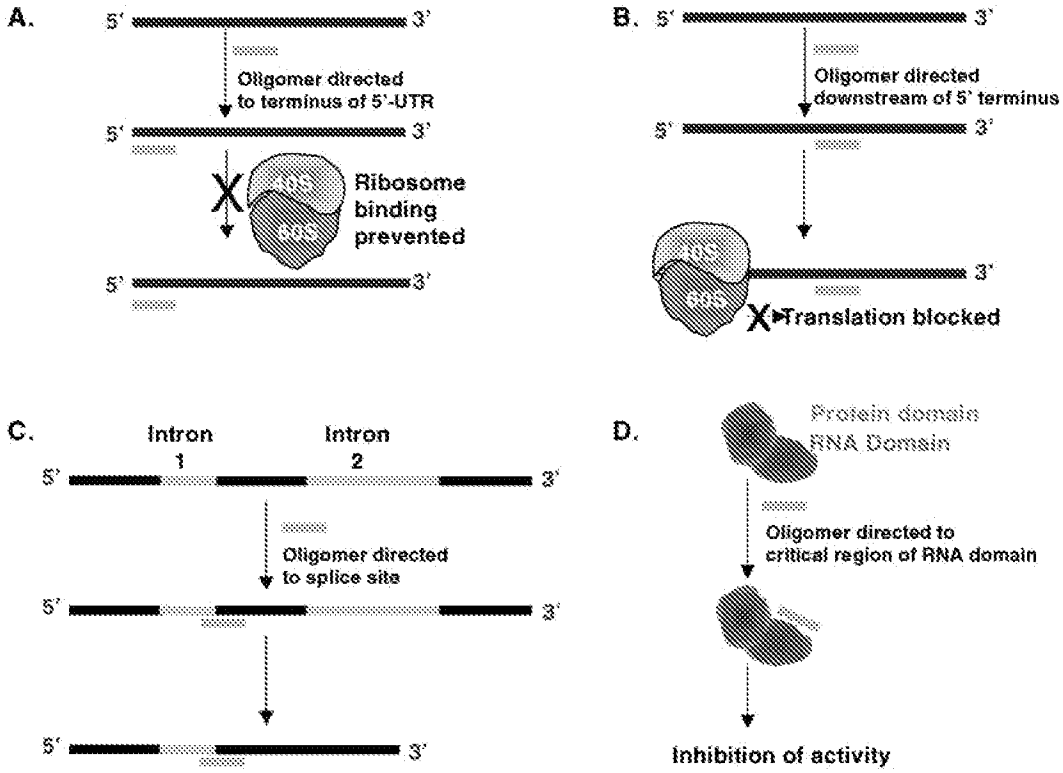

FIGURE 2: Blocking nucleic targets by antisense oligonucleotides. (A) Inhibition of gene expression by preventing ribosome binding. (B) Inhibition of gene expression by preventing translocation of the ribosome. (C) Alteration of splicing. (D) Inhibition of ribonucleoprotein activity.

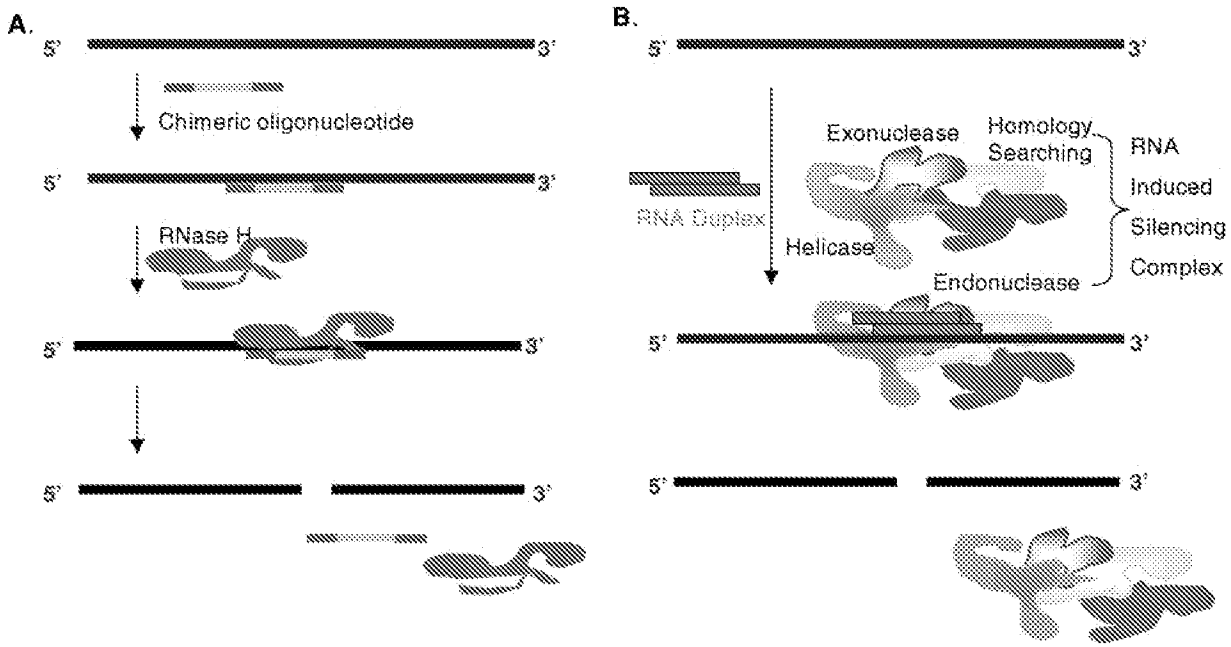

FIGURE 3: Inhibition of gene expression through recognition and destruction of mRNA. (A) RNase H and (B) RNAi-mediated cleavage of mRNA. The exact composition of the RNA-induced silencing complex is not known.

least six DNA bases can form RNA−DNA hybrids that can be recognized and cleaved by RNase H (*4*, *11*, *12*) (Figure 3A). The target RNA is destroyed, so there is no need for the oligomer to directly interfere with the binding or progression of the ribosomal complex. More recently, it has been shown that 21 or 22 base duplex RNA oligomers can interfere with mammalian gene expression (RNAi, Figure 3B) (*5*, *6*), and the implications of this phenomenon will be described below.

ANTISENSE APPLICATIONS

Antisense Drugs. Clinical development presents the pinnacle for the capabilities of the scientist to accurately predict and design useful molecules for antisense technology because potency, specificity, target selection, and pharmacokinetic properties must be optimal. One PS-DNA oligomer, Fomivirsen, was developed by ISIS Pharmaceuticals and has been approved for treatment of cytomegaloviral-induced

Table 1: Oligonucleotides Approved or in Clinical Development

oligo/target/company	disease	status
Fomivirsen/CMV IE2/ISIS	CMV retinitis	approved
Genasense/Bcl2/Genta	cancer	phase III
ISIS-3521/PKC-a/ISIS	cancer	phase III
ISIS 2302	psoriasis/Crohn's disease	phase II/III
ISIS 14803	hepatitis C	phase II
ISIS-5132/C-raf/ISIS	cancer	phase II
ISIS-2503/Ha-ras/ISIS	cancer	phase II
ISIS-104838	Crohn's disease	phase II
Gem 230/PKA/Hybridon	solid tumors	phase II
Gem132/CMVUL36/Hybridon	CMV retinitis	phase I
GEM92/HIV/Hybridon	AIDS	phase I
INX3280/Myc/INEX	restenosis	phase II
MG 98/DNA methyl transferase/MethylGene	solid tumors	phase II

retinitis (*19*). The successful completion of this clinical trial was a landmark achievement for the field, but Fomivirsen is administered by direct injection into the eye so the ability of oligonucleotides to act systemically and be applied to a wider range of diseases has been unclear. To test the broader usefulness of oligonucleotides, several clinical trials are ongoing (Table 1). These trials are possible because advances in process chemistry and purification have lowered the cost of oligonucleotide synthesis (about $200 per gram when synthesized on a kilogram scale). Oligonucleotides are usually administered to humans in doses ranging from 2 to 6 mg/kg per day, so a systemic administration over an extended period is not prohibitively expensive (*8*, *15*).

Recently, two trials have generated favorable preliminary findings that have substantially increased optimism about antisense as a general therapeutic approach. Genasense, which is being developed by Genta and targets Bcl-2, has shown encouraging signs of efficacy in trials against a variety of cancer types (*16*). Another oligomer, ISIS 3521, is being developed by ISIS Pharmaceuticals in collaboration with Eli Lilly Pharmaceuticals to target protein kinase C-α and has demonstrated favorable results in a phase I/II trial against nonsmall cell lung cancer (*15*). These preliminary results are encouraging because they indicate that oligonucleotides can enter human tumors in vivo and have a favorable impact on disease progression. Since the pharmacokinetic properties of oligonucleotides are likely to be similar regardless of oligonucleotide sequence, progress in the Genasense and ISIS 3521 trials encourages the belief that it will be possible to target many other genes successfully. It is not yet certain that these compounds are exerting their effects through the intended antisense mechanism, and providing evidence to support targeted effects in humans will present a challenge for biochemists and clinicians (Table 2).

Functional Genomics. Genome sequencing has revealed the identities of thousands of proteins, and gene expression arrays are providing a powerful tool for probing the cellular consequences of perturbing their function. The discovery of agents that can modulate the expression of these proteins would be invaluable, but how will it be possible to quickly identify the thousands of agents needed to study the multitude of new targets? Potential solutions include genetic knockouts, antibodies that can block the action of cell surface proteins, and small molecules identified by screening combinatorial or natural product libraries (*20*). All of these strategies are valuable, but important advantages for antisense oligonucleotides are that they can be synthesized as fast as sequencing information can be obtained and they belong to a class of compounds that has already produced a drug.

Table 2: Goals for Improving Oligonucleotides and Their Applications

Improve pharmacokinetics, tissue distribution, and targeting
Provide evidence that favorable clinical results are due to intended antisense targeting
Exploit high-affinity binding (PNA, LNA, etc.) to improve existing applications and develop new ones
Characterize the mechanism of RNA interference and its full potential for inhibition of gene expression for cell culture studies
Use RNAi for in vivo inhibition of mammalian gene expression
Improve the efficiency and generality of recognition of duplex DNA
Improve the efficiency of specific gene mutation
Focus more attention on RNA targets in cells other than mRNA
Perform comparative studies to demonstrate the relative strengths of different oligomer chemistries for given applications (i.e., LNA versus PNA, PNA versus morpholino, morpholino versus RNAi)
Characterize mechanism for morpholino interactions to explain their effectiveness despite the lack of enhanced affinity relative to other oligomer chemistries
Use oligomers to probe nucleic acid structure, expression, and movement in cells

Progress toward using oligonucleotides as a routine tool for inhibiting gene expression has been slowed because it is often difficult to predict mRNA sequences that will be susceptible targets for antisense inhibition. Typically only 10−20% of oligonucleotides tested will effectively block gene expression, and because it is difficult to predict how successful individual oligomers will be, it is often necessary to screen up to 20 oligomers (*2*). High throughput screens for effective oligonucleotides have been designed, and a database of effective antisense oligomers is being developed to encompass inhibitors for up to 10000 genes (www.genetrove.com). An exciting complement to the ability to systematically inactivate hundreds or thousands of genes is the ability to gain a genome-wide view of the consequences using gene expression arrays. In one of the first examples of this approach, Cho-chung and co-workers have examined the genomic effects of antisense inhibition of expression of protein kinase A RIα (*21*). This study revealed that the expression of many genes was altered, producing an expression "signature" that should allow identification of subtle or unexpected effects of inhibition of the target gene.

In Vivo Mutagenesis. Therapy for genetic disease presents one of the most difficult problems for medicine because standard treatments are unable to correct the underlying defects. Therefore, much interest has been focused on the ability of oligonucleotides to correct mutations in vivo (*22*). This is not an antisense application because the target is chromosomal DNA. However, many of the same considerations of oligonucleotide chemistry and specificity discussed above apply, and consideration of the challenges facing the targeting of DNA offers a useful perspective on the challenges facing RNA recognition. Targeted mutagenesis of genes within mammalian, plant, and yeast cells has been achieved using chimeric oligonucleotides that are either circular (*22*) or linear (*23*, *24*). Another approach for introducing genetic changes is to use triplex-forming oligonucleotides to direct mutations at specific target sites (*25*).

Laboratory applications include the targeted mutagenesis of cellular proteins, which would represent a powerful strategy for functional genomics, while clinical applications might represent a way to treat otherwise intractable genetic diseases. This area remains the subject of significant controversy (*26*, *27*), but mutagenesis frequencies ranging from 0.1% up to 20% have been reported. Chemical optimization of oligonucleotides and a better understanding of the biology of gene repair should encourage more widespread use of this technique.

Alteration of Splicing. Alternative splicing allows a single mRNA to code for the expression of multiple proteins. In contrast to the typical role for an antisense oligomer, inhibition of protein expression, by blocking one splice site an antisense oligonucleotide, can increase the expression of an alternatively spliced protein variant (Figure 2C). Since the mRNA is being translated, the oligomer must avoid activation of RNase H, making non-RNase H capable chemistries such as PNA, morpholino, or 2′-modified RNA ideal for this application.

Kole and colleagues have shown that 2′-*O*-methyl oligonucleotides directed against an alternative splice site in luciferase mRNA can cause the expression of functional luciferase to be upregulated (*28*). Not only does this work demonstrate the power of oligonucleotides to redirect splicing, it also provides a positive output for testing whether an oligomer enters a cell and finds its target. Because the output is expression instead of repression of an activity, the observed effect can be much more easily certified as a legitimate antisense interaction and provides a useful positive control for any antisense experiment. Antisense oligonucleotides have been used to redirect the splicing of several genes of therapeutic interest, including Bcl-x (*29*) and dystrophin (*30*).

Inhibition of Telomerase. Telomerase is a ribonucleoprotein that contains an RNA domain that binds to telomere ends and a protein domain responsible for maintaining telomere length from one generation to the next. Telomerase is expressed in cancer cells but not in adjoining normal tissue, leading to the hypothesis that telomerase inhibition will lead to reduced tumor cell proliferation. For the RNA domain to function it must hybridize to the telomere, suggesting that it should also be accessible to synthetic oligomers and an ideal target for inhibitor design. This hypothesis has proven to be correct, and peptide nucleic acids (PNAs) and 2′-*O*-alkyl RNA oligonucleotides (Figure 1) have been demonstrated to inhibit telomerase, cause telomere shortening, and reduce cell proliferation (*31*). Because the mechanism of action is competitive inhibition of an enzyme active site, RNase H activation is not required. Other cellular RNA molecules and ribonucleoproteins involved in signaling or enzyme activity may be equally good targets for antisense inhibition and may represent a largely overlooked set of antisense targets (Figure 2D).

NEW OPTIONS FOR ANTISENSE

Probing Development with Morpholino Oligonucleotides. Morpholino oligonucleotides are a nonionic DNA analogue available from Gene Tools LLC (www.genetools.com) (*32*). Morpholino oligonucleotides possess altered backbone linkages relative to the phosphodiester backbone of DNA or RNA (Figure 1). Complementary duplexes between RNA and morpholinos do not activate RNase H. They inhibit gene expression by a steric mechanism provided the inhibitor is targeted to the region encompassing the 5′-UTR through about +20 of the transcript (*32*). The strength of hybridization of morpholino oligonucleotides is similar to analogous DNA oligomers and much less than analogous PNAs, necessitating use of relatively long 25 base oligomers for antisense applications. Due to the neutral backbone, morpholino oligomers are less likely to form undesired interactions with cellular proteins, especially when used at high concentration (*32*).

Selective control of gene expression by antisense oligonucleotides early in development would be a powerful tool for probing growth and differentiation. Recent reports indicate that morpholino oligonucleotides are a general approach for achieving this goal (*33*). For example, morpholino oligonucleotides microinjected into zebrafish, sea urchin, or xenopus embryos block gene expression and exert effects during the early stages of development. A full journal issue has been devoted to developments in this area (*Genesis*, volume *30*).

The high success rate for inhibition of gene expression by morpholino oligonucleotides is puzzling because the dogma has been that targeting the AUG start site is unlikely to be a general approach to gene inhibition. Why do morpholinos appear to be more effective than other antisense chemistries? The answer is unknown, but the dramatically altered neutral morpholino backbone may allow the molecule to invade local RNA secondary structure more efficiently, making accessibility less of an obstacle than it is for other types of oligomer. It is also possible that the morpholino backbone presents a more disruptive conformation to the translocation of the ribosomal complex as it scans for the AUG start site or that the requirement for longer morpholino length confers an additional degree of sequence specificity in the sequences flanking the AUG codon.

Peptide Nucleic Acids. Peptide nucleic acid (PNA) is a DNA/RNA mimic in which the phosphate deoxyribose backbone has been replaced by uncharged *N*-(2-aminoethyl)-glycine linkages. Nucleobases attached through methylene carbonyl linkages to the glycine amino group (*34*, *35*) (Figure 1) recognize complementary sequences by standard Watson−Crick pairing (*36*). Because PNAs have a neutral backbone, hybridization is not affected by the intrastrand repulsion and occurs with enhanced affinity (*36*) and rates of association (*37*). PNAs do not appear to be substrates for nucleases or proteases (*38*), and absence of a repetitive charged backbone also prevents PNAs from binding to proteins that normally recognize polyanions, avoiding a major source of nonspecific interactions (*39*).

PNAs can be readily synthesized by manual or automated methods (*40*, *41*) similar to standard procedures used to make peptides. Alternatively, full-length PNAs can be obtained from Applied Biosystems (www.appliedbiosystems.com). The commonality of PNA and peptide synthesis simplifies attachment of peptides designed to augment PNA function. Attached peptides can promote strand invasion (*42*), recruit transcription factors (*43*), and increase permeability of cellular membranes (*44*−*46*). PNAs are less soluble than DNA or RNA oligonucleotides, but simple precautions reduce their potential for aggregation (*47*).

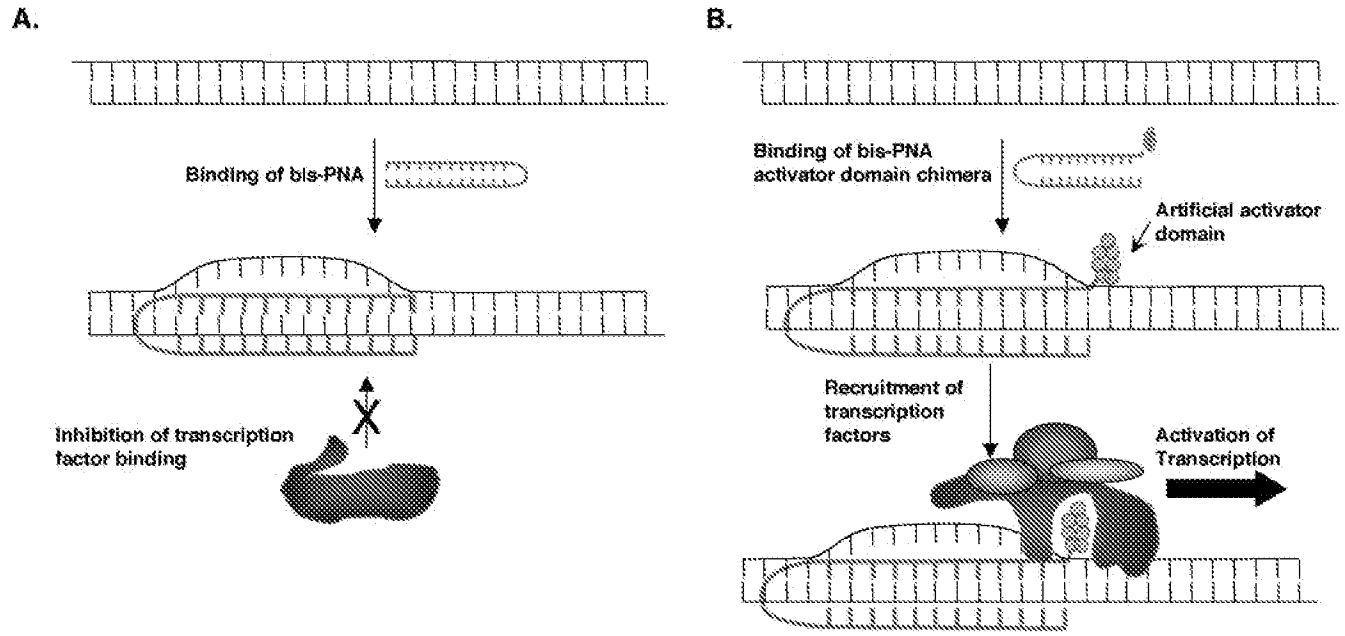

FIGURE 4: Applications of strand invasion by PNAs. (A) Inhibition of gene expression. (B) Activation of gene expression by PNA−peptide conjugates. An artificial activation domain could be a peptide, a small molecule, or any other synthetic construct capable of selectively recruiting transcription factors.

PNAs do not readily enter cultured cells unless present at high concentrations in the media (*48*), and unlike DNA or RNA, they cannot be complexed directly with cationic lipid because they are uncharged. However, it is possible to achieve intracellular delivery by first annealing a PNA to a negatively charged oligomer and adding cationic lipid to promote uptake of the hybrid (*49*). Cellular uptake of PNAs can also be achieved by the attachment of peptide sequences that promote translocation across cell membranes (*44*−*46*). The pharmacokinetic properties of PNAs in vivo are less promising than for phosphorothioate-containing DNA or 2′-*O*-alkyl RNA−DNA chimeras because clearance is rapid (*50*) and improving these properties represents an important avenue for research.

PNAs as Antisense Agents. PNA−RNA hybrids are not substrates for RNase H (*51*). As a result, to affect gene expression, antisense PNAs must use a steric blocking mechanism (Figure 2). Our laboratory has examined gene inhibition by PNAs targeted throughout the mRNA encoding luciferase, and we have observed that only PNAs complementary to the 5′ terminus of the untranslated region (*49*) inhibit gene expression. Other laboratories working with cell-free extracts or in vivo have reported that antisense PNAs can effectively target a broader range of mRNA sequences (*45*, *46*, *52*, *53*). This discrepancy may reflect the fact that the concentration of PNA needed to inhibit different genes will vary, possibly depending on the expression level of the gene of interest or the secondary structure of the target mRNA. Conflicting results reinforce the need to follow the stringent guidelines developed to validate traditional antisense approaches (*9*).

Strand Invasion by PNAs. While PNAs appear to act as antisense agents, there is no evidence that they hold advantages relative to chemically modified antisense DNA or RNA oligomers. Instead, the unique strength of PNA may lie in recognition of duplex DNA (*54*). Strand invasion of supercoiled DNA can occur at mixed sequences (within supercoiled DNA) (*37*, *42*), while strand invasion within relaxed DNA by polypyrimidine PNAs can occur through formation of a four-stranded complex in which one PNA strand binds by Watson−Crick pairing and a second binds by Hoogstein base pairing (*54*, *55*).

Strand invasion by polypyrimidine PNAs can be enhanced by use of bis-PNAs in which the two hybridizing strands are tethered by a flexible linkage because attachment of the two PNAs reduces the entropic penalty of binding (*56*). Nielsen and co-workers have also shown that pseudocomplementary PNAs containing diaminopurine−thiouracil base pairs can be used to target a broad range of duplex sequences (*57*). Our laboratory has observed that the efficiency of strand invasion by bis-PNAs at polypyrimidine sites can be greatly enhanced by attaching the PNA to a short cationic peptide (Kaihatsu, unpublished). If robust protocols can be developed to achieve strand invasion of chromosomal DNA within cells, improved applications for PNAs might include inhibition of gene expression (Figure 4A), activation of gene expression (Figure 4B), induction of mutations, and probing chromosome topology.

LNAs: Potential Applications of Exceptionally High Affinity Hybridization. Locked nucleic acid (LNA, also known as bridged nucleic acid, BNA) bases are RNA analogues that contain a methylene bridge connecting the 2′-oxygen of the ribose with the 4′-carbon (Figure 1). This bridge results in a locked 3′-endo conformation, reducing the conformational flexibility of the ribose and increasing the local organization of the phosphate backbone (*58*, *59*). This design confers outstanding affinity to complementary hybridization, with melting temperature values being increased by up to 10 °C per substitution (summarized in ref *60*). Oligomers that contain LNA bases can bind with higher affinity than analogous PNAs (Braasch, unpublished) and represent an exciting option for high-affinity binding. LNA bases are introduced by standard DNA/RNA synthesis methods, and DNA−LNA hybrids can be readily obtained. This synthetic flexibility encourages the tailoring of existing

antisense oligonucleotides to improve their properties and suggests that the use of LNA bases might be a simple strategy for enhancing activity. LNA bases are not available commercially, but LNA oligomers and LNA−DNA chimera can be purchased commercially from Proligo LLC (proligo.com) or Cureon (cureon.com).

Like other 2′ modifications, the 2′−4′ linkage reduces or eliminates activation of mRNA cleavage by RNase H (*61*). However, because LNA bases are added by standard DNA/RNA synthesis protocols, it is straightforward to design chimeric "gapmers" in which a central DNA portion is flanked by LNA intended to enhance the stability of binding. Such chimera allow the high affinity of LNA binding to be combined with the ability of DNA to recruit RNase H (Figure 3A). It is particularly attractive to envision that the addition of a limited number of LNA bases to antisense oligomers that are already known to block gene expression might significantly improve the potency and specificity of their action.

Few studies on the uses of LNA have been published, but Wengel and co-workers report that LNA−DNA chimera targeted to DOR, a G protein-coupled receptor, produce a physiological response in mice when injected directly into the brain (*61*). Very recently, Wengel and Gait have shown that LNA/2′-*O*-meRNA chimera targeted to a sequence within the HIV-1 transactivating responsive element (TAR) RNA can block the binding of protein factors and inhibit transcription in cell extract (*62*).

RNAi. RNA interference (RNAi) is a new approach to antisense gene inhibition that makes full use of an ancient cellular mechanism for silencing of viral sequences. In 1998 Fire and co-workers demonstrated that relatively long double strands of RNA were remarkably potent and selective inhibitors of gene expression in *Caenorhabditis elegans* (*63*). Subsequently, Tuschl showed that the long RNAs were processed into 21 or 22 base oligomers inside cells and that short synthetic RNA oligonucleotides could be transfected into mammalian cells (*5*, *6*; reviewed in ref *64*). Early reports indicate that mammalian RNAi is remarkably effective. Gene inhibition is efficient, non-sequence-selective toxicity is low, and a high percentage of duplexes produce the desired inhibition. As a result, RNAi will likely offer new opportunities for using antisense oligomers in basic research. While the pharmacokinetic properties of duplex RNA are currently unknown, if good in vivo uptake can be achieved, the use of RNAi might significantly improve the ability of oligonucleotides to have an impact in animal studies and drug development.

Future of Antisense in Basic Science and Medicine. The use of oligonucleotides to control gene expression has long fascinated researchers because of the potential to rapidly generate potent and specific agents. In the past, antisense technology has not always kept pace with expectations, but recent advances in diverse areas are likely to make it a routine and trusted research tool. It should be emphasized that the powerful new options for using oligonucleotides to control gene expression do not reduce the need for use appropriate control experiments (*9*) and that these need to continue to be an integral part of any study that aims to be persuasive.

Genome sequencing has revealed the identities of thousands of antisense target sequences, and new oligonucleotide chemistries, such as PNA, LNA, morpholino, and 2′-*O*-alkyl RNA, are available that possess chemical properties that substantially improve the potency of target recognition. Experience in the clinic is demonstrating that even older generation oligonucleotide designs are effective drugs, and a detailed database of pharmacological information is being developed. This advance of basic and clinical knowledge suggests that many opportunities exist for chemists and biologists to continue improving recognition by oligonucleotides (Table 2), and it is likely that the field will continue to generate exciting new options for controlling gene expression and probing cell biology.

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Due to space constraints, we are unable to cite all relevant work or describe every promising oligonucleotide chemistry. We apologize to our colleagues for this.

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